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(54) Title: PEPTIDE

(57) Abstract

The present invention relates to a new method of delivery of molecules into a cell through the use of a modified signal peptide to which a peptide nucleic acid is linked. The signal peptide will comprise at least one positively charged amino acid residue, or functional equivalent thereof. The addition of such positively charged residues can serve as a linker group for the attachment of peptide nucleic acids to the signal peptide thus increasing the number of peptide nucleic acid sequences delivered by the signal peptide and therefore its functional efficiency. Extension of the signal peptide at the C or N terminus through the addition of a single or multiple charged residue or analogues thereof will modify and improve signal peptide delivery function by increasing the solubility and cell permeability characteristics of the signal peptide.

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"Peptide"

The present invention relates to the delivery of molecules into a cell and the use of modified signal peptides.

Specifically, a modified analogue of the signal peptide sequence from Karposi syndrome fibroblast growth factor (kFGF) is used as a cell-permeant vehicle for the intracellular delivery of covalently linked anti-sense peptide nucleic acid sequences (PNAs).

PNAs have potential uses as antisense molecules for the control of gene expression. Since they are capable of binding tightly to DNA and RNA targets thus preventing DNA transcription to RNA and RNA translation to protein. These molecules thus have two potential uses of commercial importance:

1. As research reagents where scientists use antisense strategies to ablate selected genes in order to understand their function.

2. As pharmaceutical compounds for companies seeking to develop nucleic acid-based therapies.

Conventional anti-sense oligonucleotide *in vivo* delivery is highly inefficient, even if long-lasting, less polar phosphorothioates are used.

This invention covers the use of cell-permeant peptide delivery systems based on the hydrophobic core sequences of any signal peptide sequence. A signal peptide is a short-lived *N*-terminal sequence found only on nascent proteins which are synthesised in the endoplasmic reticulum. Signal peptides consist of three domains:

(a) *N*-terminus of 1-5 amino acids, often positively charged;

(b) A hydrophobic core or central region (7-16 amino acids) which is essential for translocation across the endoplasmic reticulum membrane; and

(c) A more polar C-terminal domain (3-7 amino acids) which is important for specifying the cleavage site.

Synthetic peptides consisting of only the hydrophobic cores are typically insoluble in water. Taking the signal peptide sequence of Karposi syndrome-derived FGF as an example, we have modified these insoluble sequences by the addition of positively charged amino acids (for example lysines), which have the effect of rendering them water soluble without compromising their ability to translocate across cellular membranes. The ability to add amino groups in this way allows extra cargo sequences to be conjugated to these amino groups.

It is an object of the present invention to provide a cell permeable peptide delivery system based on a

signal peptide sequence for the intracellular delivery of peptide nucleic acid sequence.

According to the present invention there is provided a cell permeable peptide comprising at least the hydrophobic core of a signal peptide or an analogue thereof wherein the peptide is modified by the addition of at least one positively charged amino acids or positively charged analogues thereof.

The signal peptide may be a natural or synthetic signal peptide or a peptide which is substantially similar thereto.

A peptide which is substantially similar to a signal peptide is at least 60% homologous thereto.

At least one positively charged amino acid is chosen from lysine and/or arginine and/or any positively charged analogues thereof.

In one particular embodiment the cell permeable peptide is a modified analogue of Karposi syndrome fibroblast growth factor (kFGF).

The positively charged amino acid consists of one or more lysine residues.

The invention further provides the use of cell permeable peptides as described herein for intracellular delivery of a molecule.

Preferably, one or more lysine residues will be attached to the C terminal of the signal sequence peptide or signal sequence peptide analogue.

This positively charged lysine allows the linkage of a peptide nucleic acid, thus facilitating in vivo delivery of the said peptide nucleic acid.

The invention also provides a cell permeable peptide which contains multiple positively charged amino acids or positively charged analogues thereof wherein a peptide nucleic acid may be conjugated to each positively charged residue and wherein the peptide nucleic acids conjugated by such a means are identical or different.

The invention also provides a cell permeable peptide which comprises at least one positively charged amino acid residue or functionally equivalent positively charged analogue thereof conjugated or conjugatable to a lysine tree, to which multiple peptide nucleic acids may be joined for transport and presentation.

The linked peptide nucleic acid sequence may be antisense.

Preferably, the peptide nucleic acid sequence will be covalently linked.

The present invention thus allows the use of cell permeable peptides as described herein to deliver peptide nucleic acids to in-vivo targets.

Use of conventional oligonucleotides is being reduced due to the development of PNAs (Neilsen, et al., 1991), which are much more stable, being resistant to enzymic degradation (Jordan, et al., 1997). PNAs replace the phosphodiester backbone of nucleic acid with repeating N-(2-aminoethyl)glycine units to which natural

nucleobases are attached through methylenecarbonyl linkers. Although more stable, PNAs suffer from similar accessibility problems as phosphorothioates do, and passive diffusion of unmodified PNA across lipid membranes is not efficient (Wittung, P., et al., 1995).

A small number of native peptide sequences can translocate across membranes of living cells in an energy-independent and receptor-independent manner. These peptides have been used to import active cargo into the cell. For example a peptide from the homeodomain of *Antennapedia* has been successfully used to import both peptidal inhibitors of protein kinase C (Theodore, et al., 1995) and conventional anti-sense oligonucleotides (Allinquant, et al., 1995).

The present invention provides use of cell permeable peptide import (CPPI) to deliver peptide nucleic acids (PNAs).

The present invention provides use of the signal peptide sequence from Karposi syndrome fibroblast growth factor (kFGF) for delivery of antisense peptide nucleic acid sequences (PNAs).

The invention provides use of a peptide as defined herein together with lysine residues for multiple presentation of peptide nucleic acids.

The invention further provides use of peptides as defined herein together with lysine residues in the simultaneous presentation of different peptides nucleic acids.

The present invention combines the two above technologies to use CPPI to deliver PNAs to *in vivo*

targets.

The invention described herein has the following advantages:

- The modified signal peptides described in this invention can be used for the delivery of any cell-impermeant substance into cells.

- The signal peptides described in this invention can be used to improve the delivery of substances of low permeability into cells.

- The delivery of substances to particular cellular sub-compartments can be achieved and improved by incorporating appropriate targeting peptide sequences or other modifications to the signal peptides. Effects are only due to the 'cargo' substance that they carry. For example, addition of a myristoyl moiety to the peptide would ensure that it was preferentially retained at the plasma membrane.

- The signal peptide delivery system has commercial value in therapeutic drug-delivery systems including, but not restricted to, gene therapy, cancer therapy and anti-infectious agent therapy.

- This system also has commercial value as a tool for biochemical and molecular biological research.

- The modified signal peptides described in this invention do not, themselves, exhibit any biological effects nor do they affect cell viability. Effects are only due to the 'cargo' substance that they carry.

This invention will be exemplified in the following non-limiting examples with reference to the accompanying figures wherein:-

Figure 1 illustrates carboxyfluorescein labelled kFGF signal peptide-Lys.Lys.Lys - fluoresence calibration curve.

Figure 2 illustrates carboxfluorescein labelled cell permeant peptide incorporation by whole human endothelial cells.

Figure 3 depicts incorporation of carboxyfluorescein labelled signal peptide-Lys.Lys.Lys by cell.

Figure 4 illustrates subcellular distribution of labelled signal peptide in cells.

Figure 5 depicts incorporation of labelled kFGF peptide into human dermal endothelial cells.

Figure 6a sets out the signal peptide sequence and modifications.

Figure 6b illustrates simultaneous presentation of 3 PNAs directed to different sites on a target RNA.

Figure 6c illustrates multiple presentation of the single PNA species.

Table 1 describes carboxyfluorescein derivatised cell permeant peptides.

Table 2a sets out uptake of cell permeant peptides by cells.

Table 2b sets out cellular uptake of permeant peptides by BHK cells.

Table 3 sets out results of washing labelled antennapedia cells.

Table 4 sets out washing results for labelled signal peptide-KKK and cells.

EXAMPLE 1

This is an example of the intracellular delivery of a low molecular weight compound (carboxyfluorescein) which is normally cell impermeant.

In order to determine the best delivery system, a comparison of the ability of four different cell permeant peptides (Table 1) to accumulate in whole cells was undertaken. The four people peptides were synthesised to contain carboxyfluoresein as a reporter group (Table 1), allowing intracellular accumulation to be monitored by fluorescence. Whole cells were exposed to 50 μM solutions of each peptide for 24 hours (37°C) and accumulation was measured using a fluorometer. The results of this are shown in Tables 2A and 2B.

The results shown in the whole column of Table 2A were provided by cell suspensions being exposed to 50μM peptide each, for 24 hours at 37°C. Incubations contained 3.28 x 106 cells in 1 ml. Subcellular fractionation was then carried out. Fluorescence measured with excitation λ = 471 nm, emission λ = 521 nm. RFU valves were converted to nMoles per 10^6 cells.

The raw relative fluorescent units (RFU) values were converted to nMoles per 10^6 cells using a calibration

curve constructed for each peptide. An example of a fluorescence calibration curve of fluorescein labelled kFGF is shown in Figure 1.

The kFGF-KKK sequence (see Figure 3) shows similar high rates of cytosolic and nuclear incorporation compared with the antennapedia peptide (Table 2A). The PKC and substance P peptides show much lower incorporation Table 2A & 2B). Incorporation of the kFGF-KKK sequence is saturable, as can be seen from the data presented on Figure 2 and time-dependent as shown in Figure 3.

Table 2A shows that antennapedia is lost during subcellular fractionation. Unlike the antennapedia peptide, carboxyfluorescein-kFGF signal peptide-KKK is not loosely attached to the cell surface as shown in Tables 3 and 4. Unlike the antennapedia peptide, carboxyfluorescein-kFGF signal peptide-KKK does not remain membrane-bound as shown by the data presented in Figure 4.

It should be noted from Figure 4 that all cells treated with carboxyfluorescein - labelled kFGF signal peptide Lysine-Lysine-Lysine have nuclear and cytoplasmic incorporation. Unlike antennapedia, very little remains stuck in the cell membrane.

EXAMPLE 2 - Anti-sense agents for gene ablation

Conventional oligonucleotide sequences or those in which the phosphodiester bonds are replaced with nuclease-resistant bonds (such as the phosphothiorates and the like) may be conjugated to the kFGF-derived delivery system for intracellular delivery and subsequent specific blocking of gene translation or Rnase-targeted destruction of the mRNA in question.

Alternatively peptide nucleic acid sequences may be used, as in example 1.

Although the "cargo" to be delivered intracellularly is referred to in the text and represented in the accompanying figures as a Peptide Nucleic Acid (PNA), it should not be limited to such cargo type as the various configurations of CPPI described in this Patent could also be used to carry peptide sequences or oligonucleotide sequences (either native sequences or modified sequences, such as phosphothiorates).

It has been demonstrated that addition of a peptide nucleic acid sequence does not impede incorporation of the carboxyfluorescein-kFGF signal peptide-{PNA}-KKK. The confocal micrograph shown in Figure 5 illustrates this.

EXAMPLE 3

Nuclear localisation signal (NLS) sequences such as are found on transcription factors like NF-kappaB may be conjugated to the kFGF-derived delivery system, as in Example 1. Intracellular delivery of NLS peptide sequences would act as 'bait' to selectively block the translocation of the selected transcription factor, thus preventing its action. In this way, genes under the control of the transcription factor could be identified on the basis of down regulated expression.

EXAMPLE 4

Signal transduction motifs such as phosphotyrosine-containing peptide sequences (pYP's) act as docking sites for a large number of proteins. Such signalling proteins contain domains that recognise (contextually)

the phosphotyrosine residues and bind to them in a specific manner. pYP's are recognised by SH-2(Src-homology-2) domains and PTB (phosphotyrosine binding domains). Specificity is provided by short amino acid sequences *N*-and/or *C*-terminal of the phosphotyrosine. Such peptide motifs could be conjugated to the kFGF peptide-derived delivery system as in Example 1, and could be used to intracellularly deliver pYP's which would act as bait, thus allowing signal pathways to be 'interrogated'.

The signal sequence of kFGF was modified to contain three lysines at the C-terminal of the hydrophobic signal sequence. This procedure is illustrated in Figure 6A. In this Figure 6A (I) shows the signal peptide with an attached reporter group. Figure 6A Part II illustrates the addition of the tri-lysine extension to the C-terminal of the signal peptide sequence, thus providing three positive charges which aid solubility and cell permeability. In Figure 6A Part IIIb, the peptide nucleic acid forms part of the linear primary amino acid sequence, with Part IV illustrating a tri-lysine C-terminal extension to the peptide nucleic acid sequence providing 3 positive charges and aiding solubility and cell permeability.

Part V of Figure 6A further shows a tri-lysyl extension at the N-terminal of the signal peptide which provides 3 positive charges aiding solubility and cell permeability. The addition of the tri-lysyl extension proximal to the carboxyfluorescein reporter group enhances its fluorescence. In Vb of Figure 6A, the peptide nucleic acid sequence initially forms part of the linear primary amino acid sequence at the N-terminal of the original peptide, before a tri-lysyl extension is added to the N-terminal of the peptide

nucleic acid extension.

It should be noted that although the above examples specifically use the amino acid lysine for the addition of positive charge, molecules containing similar properties such as arginine or analogues thereof, of either of these molecules could also be used.

This peptide, therefore, can accommodate three PNAs, each bonded to a lysine epsilon amino group. This can be extended using the Multiple Antigen Presentation (MAP) technology to present eight (or more) PNA's on one kFGF signal sequence. A 'lysine tree' constructed in this way accommodates eight copies of the same PNA, thus increasing the effective concentration delivered by each CPPI.

An example of the addition of such a lysine tree is shown in Figure 6C Parts I-IV. In Part I a single lysine molecule added to the C-terminal of the kFGF signal peptide sequence allows the multiple PNA lysine tree to be added to the e-amino group of the lysine side chain.

Alternatively, Part II of Figure 6C a lysine molecule added to the N-terminal of the kFGF signal peptide sequence allows the multiple PNA lysine tree to be added to the e-amino group of the lysine side chain.

Part III of Figure 6C further shows that when a C-terminal tri-lysine extension is added to the signal peptide with N-terminal associated multiple PNA lysine tree, the 3 positive charges aid solubility and cell permeability of the molecule.

Part IV of Figure 6C add a tri-lysyl extension at the

N-terminal of the signal peptide which is attached to the lysine group added to allow attachment of the multiple PNA lysine tree as originally illustrated in Figure 6C Part II. The addition of the 3 positively charged molecules at this terminal of the molecule, proximal to the carboxyfluorescein reporter group enhances its fluorescence.

Alternatively a carrier can be constructed containing three (or more) different PNAs directed towards different sites on the same target mRNA. This strategy has been termed 'molecular triangulation' (Branch, A.D., 1998).

Figure 6B illustrates this process of 'molecular triangulation'. Figure 6B Part I shows the signal peptide with a C-terminal tri-lysyl extension which allows three different PNA sequences to be conjugated to the epsilon-amino groups of the three lysines.

Figure 6B Part III shows the addition of a further three lysines to the molecule of Part I, which adds three positive charges, which aid solubility and cell permeability. Figure 6B Part III shows the addition of the tri-lysyl extension to the N-terminal of the molecule of Part I. Again the 3 positive charges aid the solubility and cell permeability of the molecule, which their proximal location to the carboxyfluorescein reporter group enhances its fluorescence.

Figure 6B, Part IV, illustrates an N-terminal tri-lysyl extension added to the kFGF signal peptide sequence, which subsequently allows three different PNA sequences to be conjugated to the epsilon-amino groups of the lysines.

Further, this molecule has 3 lysines added at the C-terminal to add positive charge which aid solubility and cell permeability. Figure 6B Part V shows the signal peptide again with the three peptide nucleic acid associated tri-lysine extension at the N-terminal, but with the addition of the further 3 lysine groups also being made to the N-terminal where they will have the effect of aiding solubility and cell permeability, which also enhance the fluorescence of the carboxyfluorescein reporter group due to their proximity.

Further to the sequences illustrated in Figures 6A and 6C additional tri-lysine extensions at either end of the molecule, appears to aid solubility and cell permeability to allow PNA sequences to be transported. Therefore in addition to using lysine residues to attach to PNA sequences, additional tri-lysine extension is recommended. Examples of presentation peptide using the additional try-lysine is demonstrated in Figures 6B (II-IV), Figures 6C (III-IV) and Figures 6A (IV, IVb, V, Vb).

Lysine extensions comprising more or less than three lysine residues may also be useful to provide additional solubility and cell permeability.

The lysine extension may be provided next to a carboxyfluorescein reporter group to enhance its fluorescence.

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Neilsen, P.E., Egholm, M., Berg, R.H. and Buchardt, O. (1991) Sequence-selective recognition of RNA by strand displacement with a thymine-substituted polyamide. *Science*, 254: 1497-1500.

Theodore, L. Derossi, D., Chassang, G., Llirbat, B., Kubes, M., Jordan, P., Chneiweiss, H., Godement, P. and Prochiantz, A. (1995) Intraneuronal delivery of protein kinase C pseudosubstrate leads to growth cone collapse. J. Neurosci., **15**: 7158-7167.

Wittung, P., Kajanus, J., Edwards, K., Haaima, G., Nielson, P., Norden, B. and Malmstrom, B.G. Phospholipid membrane-permeability of peptide nucleic-acid (1995) *FEBS Lett.*, **375**: 317-320.

CLAIMS

1 A cell permeable peptide comprising at least the hydrophobic core of a signal peptide or an analogue thereof wherein the peptide is modified by at least the addition of at least one positively charged amino acid or positively charged analogue thereof.

2 A cell permeable peptide as claimed in claim 1 wherein the signal peptide is a natural or synthetic signal peptide or a peptide which is substantially similar thereto.

3 A cell permeable peptide as claimed in claim 1 and 2 wherein at least one positively charged amino acid is chosen from lysine and/or arginine and/or any positively charged analogue thereof.

4 A cell permeable peptide as claimed in any preceding claim wherein the cell permeable peptide is a modified analogue of Karposi syndrome fibroblast growth factor (kFGF).

5 A cell permeable peptide as claimed in any preceding claim where in the positively charged amino acid consists of one or more lysine residues.

6 A cell permeable peptide as claimed in claim 5 wherein one or more lysine residues are attached to the C-terminal of the signal sequence peptide or signal sequence peptide analogue.

7 A cell permeable peptide as claimed in any of claims 1 to 6 which contains multiple positively

charged amino acids or positively charged analogues thereof, wherein a peptide nucleic acid may be conjugated to each positively charged residue and wherein the peptide nucleic acids conjugated by such means are identical or different.

8 A cell permeable peptide as claimed in any of claims 1 to 6 which comprises at least one positively charged amino acid residue or functionally equivalent positively charged analogue thereof, conjugated or conjugatable to a lysine tree, to which multiple peptide nucleic acids may be joined for transport and presentation of multiple peptide nucleic acids.

9 Use of cell permeable peptides claimed in any of the preceding claims for intracellular delivery of a molecule.

10 Use of a cell permeable peptide as claimed in any of claims 1 to 8 to deliver peptide nucleic acids to *in-vivo* targets.

Figure 1

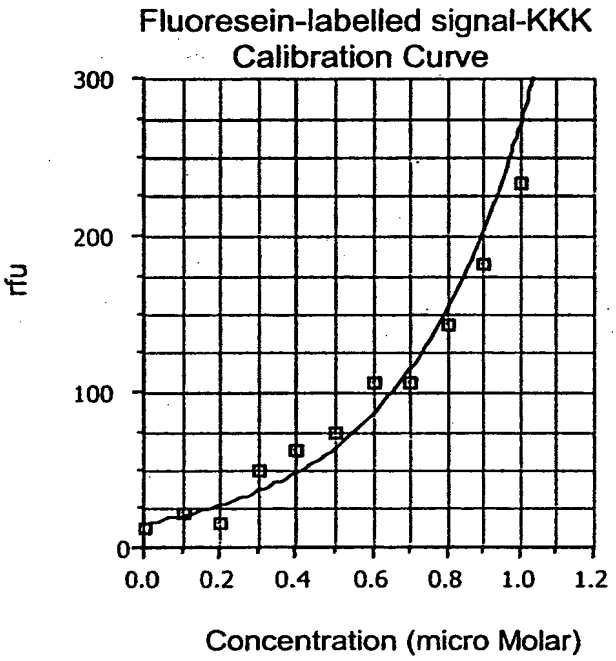

Fluoresein-labelled signal-KKK
Calibration Curve
rfu
300
200
100
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Concentration (micro Molar)

Figure 2

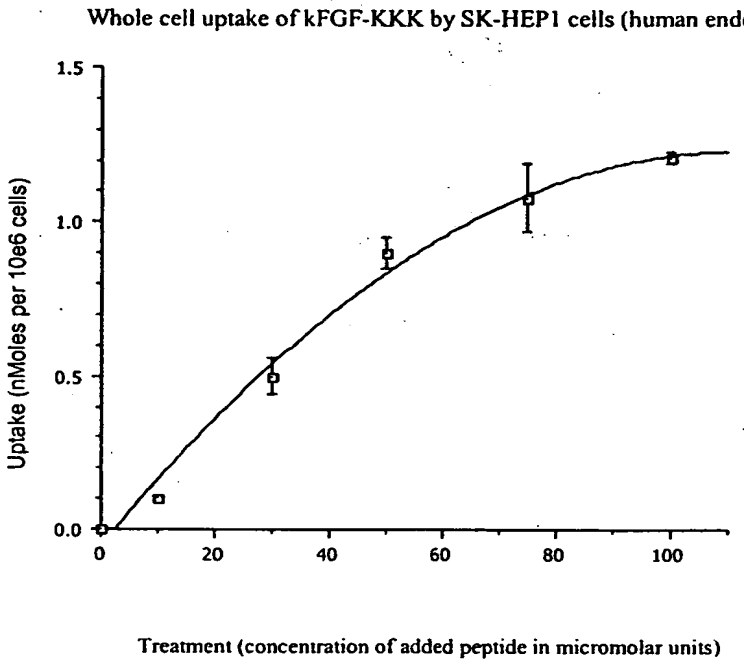
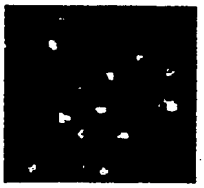
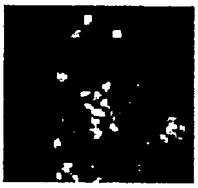

Whole cell uptake of kFGF-KKK by SK-HEP1 cells (human endothelial)
Uptake (nMoles per 10e6 cells)
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Treatment (concentration of added peptide in micromolar units)

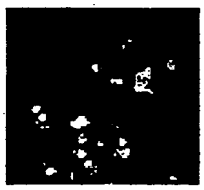
Figure 3


15 minutes


30 minutes


45 minutes


1 hour


4 hours

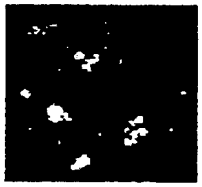

24 hours

Figure 4

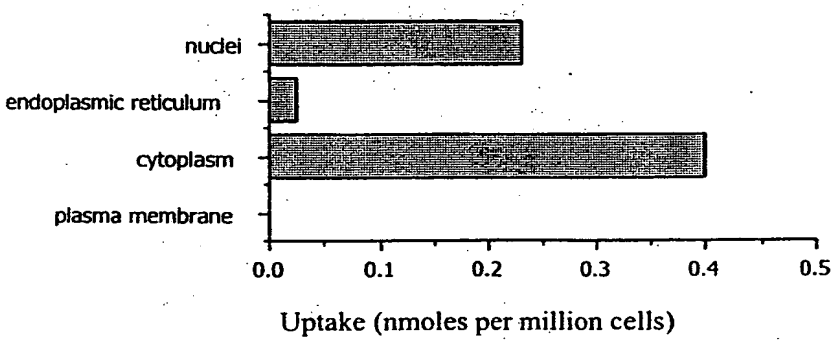

nuclei
endoplasmic reticulum
cytoplasm
plasma membrane
0.0
0.1
0.2
0.3
0.4
0.5
Uptake (nmoles per million cells)

Figure 5

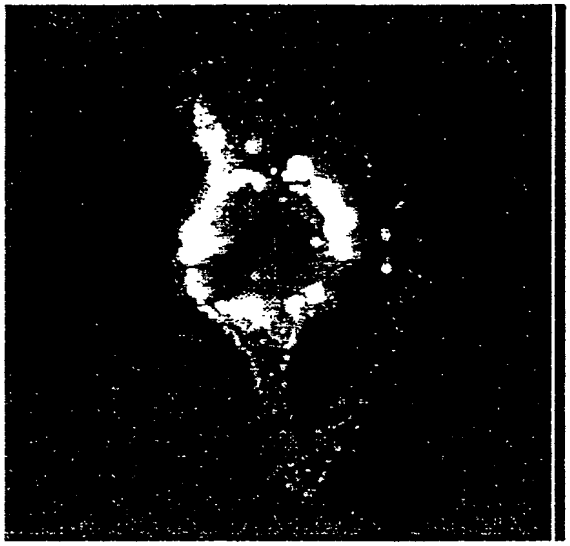


Figure 6A

A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P *6A(I).*

CarboxyFluor -A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P *6A(II)*

CarboxyFluor -A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P. K.K.K *6A(III)*

CarboxyFluor -A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P-[PNA SEQUENCE] *6A(IIIb)*

CarboxyFluor -A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P-[PNA SEQUENCE]-K.K.K *6A(IV)*

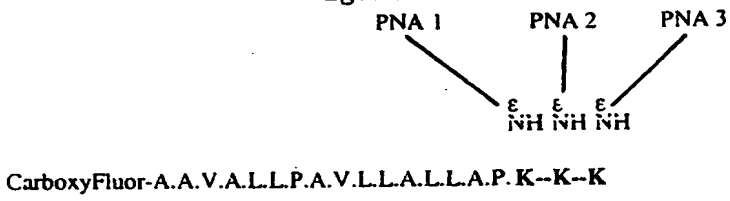
CarboxyFluor -A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P. K.K.K -[PNA SEQUENCE] *6A(IVb)*

CarboxyFluor -.K.K.K —A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P-[PNA SEQUENCE] *6A(V)*

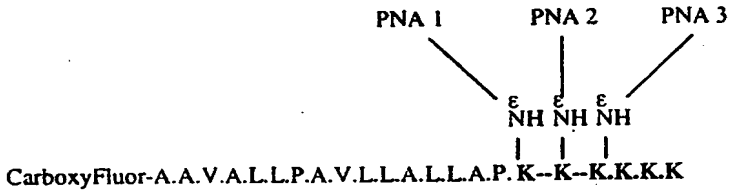
CarboxyFluor -.K.K.K -[PNA SEQUENCE]-A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P *6A(Vb)*

Figure 6B

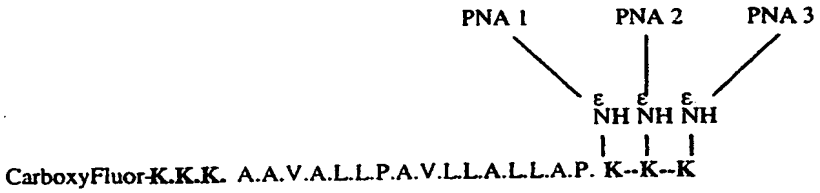
6B(I)



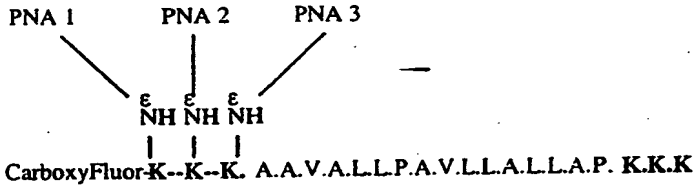
6B(II)



6B(III)



6B(IV)



6B(V)

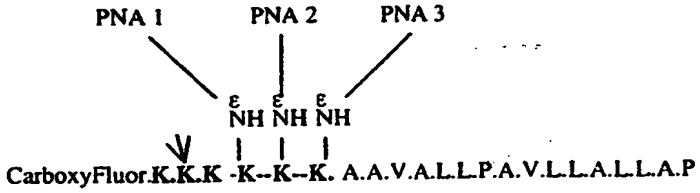
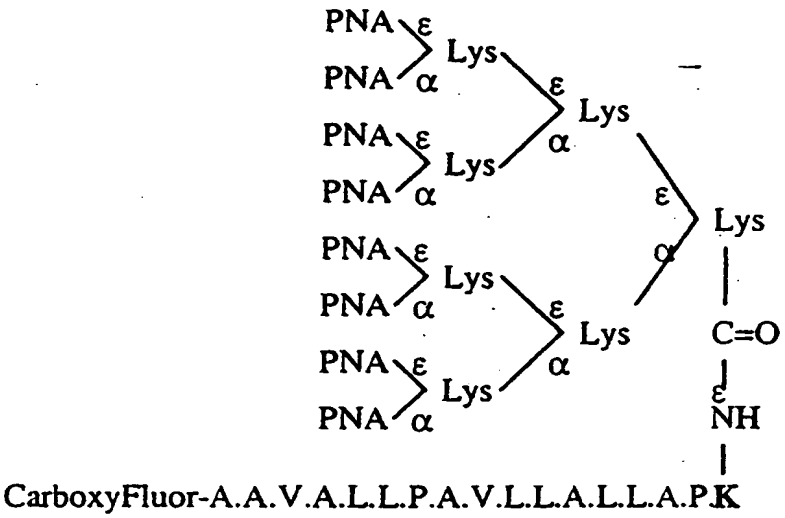
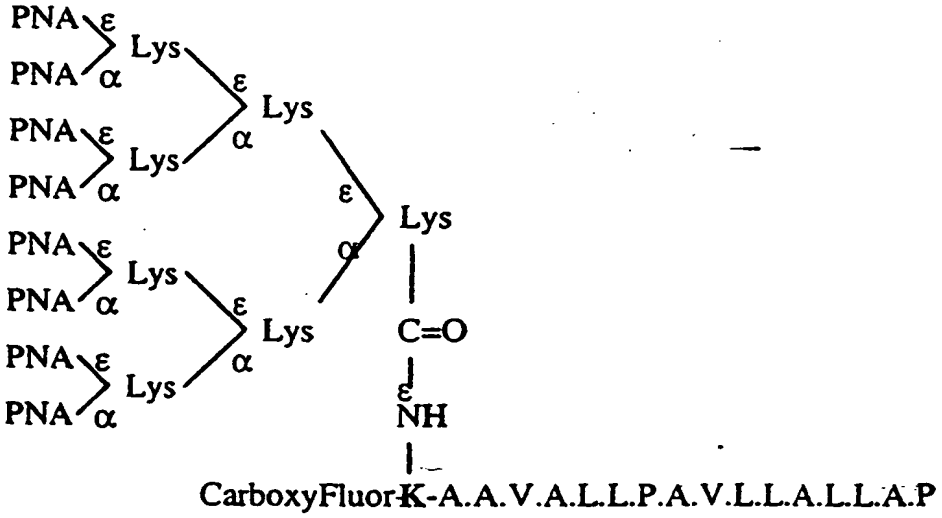


FIGURE 6C

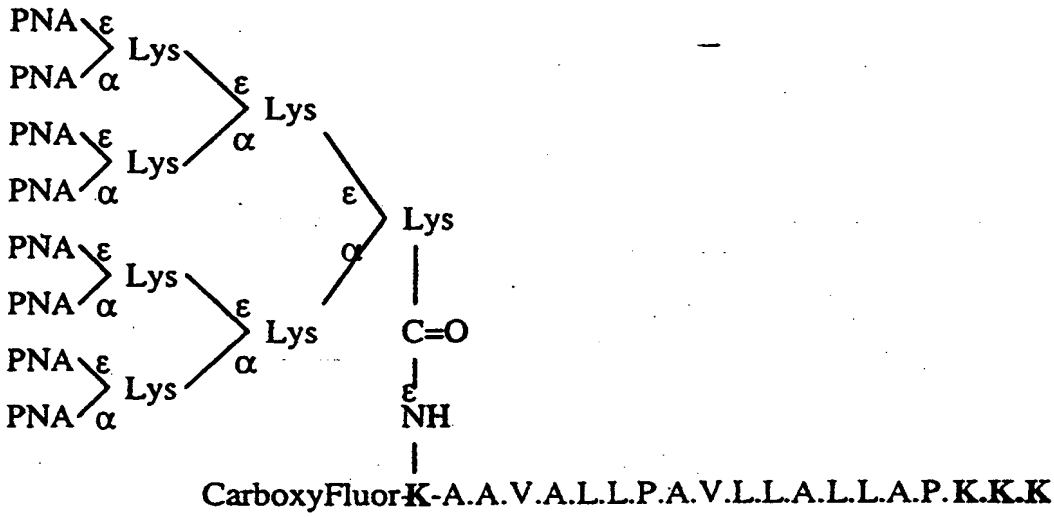
6C(I)



6C(II)



6C(III)


PNA
PNA
Lys
Lys
Lys
Lys
Lys
Lys
Lys
C=O
NH
CarboxyFluor-K-A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P.K.K.K

6C(IV)

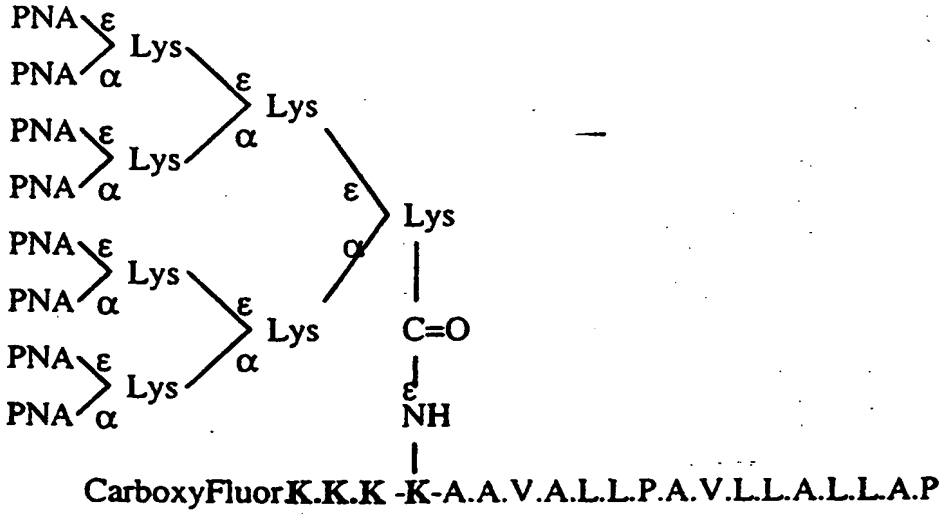

PNA
PNA
Lys
Lys
Lys
Lys
Lys
Lys
Lys
C=O
NH
CarboxyFluorK.K.K -K-A.A.V.A.L.L.P.A.V.L.L.A.L.L.A.P

Table 1

Carboxyfluorescein-derivatised Cell Permeant Peptides*																				
kFGF signal sequence	cFl	A	A	V	A	L	L	P	A	V	L	L	A	L	L	A	P	**K**	**K**	**K**
PKC Pseudo - substrate	cFl	R	F	A	R	K	G	A	L	R	Q	K	N	V	H	E	V	K	N	
Substance P	cFl	R	P	R	P	Q	Q	F	**Ø**	G	L	M								
Antennapedia	cFl	R	Q	I	K	I	W	F	Q	N	R	R	M	K	W	K	K			

*Modifications of original sequence marked in bold (ø = ornithine, cFl = carboxyfluorescein).

Table 2A

	*WHOLE CELL nmoles per 10^6 cells	CYTOSOL nmoles per 10^6 cells	NUCLEI nmoles per 10^6 cells
FGF-KKK	0.79	0.37	0.35
KKK-FGF-KKK	0.24	0.046	0.15
Substance P	0.03	0.005	0.015
PKC pseudo - substrate	0.034	0.015	0.007
Antennapedia	1.22	0.34	0.35

*Cell suspensions were exposed to 50 μM peptide each, for 24 hours, at 37°C, =471nm, emission λ = 521nm. RFU values were converted to nMoles per 10^6 cells

Table 2B

CPPI sequence tested	Amount in nuclei (nmoles per 10^6 cells)	Amount in cytosol (nmoles per 10^6 cells)	Cytosolic concentration (μM)
kFGF signal peptide	0.035	0.0567	13.5
SubstanceP analogue	0.0005	0.0018	0.42
PKC pseudosubstrate	0.0005	0.00156	0.37

Table 3

Treatment	rfu
1st PBS wash -	114
2nd PBS	57.34
3rd	21.08
4th PBS/acid wash	15.36

Table 4

Incorporation Treatment	incorporation (nmoles per 106 cells
PBS wash (after 15min exposure)	0.64
Acid Wash (15min)	0.525
PBS wash (after 24hour exposure)	0.75
Acid wash (after 24hour exposure)	0.53